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Protective Antiviral Immune Responses to Pseudorabies Virus Induced by DNA Vaccination Using Dimethyldioctadecylammonium Bromide as an Adjuvant

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To enhance the efficacy of a DNA vaccine against pseudorabies virus (PRV), we evaluated the adjuvant properties of plasmids coding for gamma interferon or interleukin-12, of CpG immunostimulatory motifs, and of the conventional adjuvants dimethyldioctadecylammonium bromide in water (DDA) and sulfolipo-cyclodextrin in squalene in water. We demonstrate that a DNA vaccine combined with DDA, but not with the other adjuvants, induced significantly stronger immune responses than plasmid vaccination alone. Moreover, pigs vaccinated in the presence of DDA were protected against clinical disease and shed significantly less PRV after challenge infection. This is the first study to demonstrate that DDA, a conventional adjuvant, enhances DNA vaccine-induced antiviral immunity.

DNA vaccines show great promise as an alternative to conventional vaccines in numerous preclinical animal models. Investigative approaches designed to enhance the efficacy of DNA vaccines are of major importance, as immunity induced by DNA vaccines is often unable to provide sufficient protection against challenge infection. It has previously been demonstrated that vaccination with pseudorabies virus (PRV) glycoprotein D (gD) DNA induced high titers of virus-neutralizing (VN) antibodies, while vaccination with gB DNA induced PRV-specific cell-mediated immune (CMI) responses, including those of $CD8^+$ cytotoxic T lymphocytes (CTLs) and memory T-helper cells (12, 47), and that the intradermal route of inoculation was superior to the intramuscular route of inoculation (48). However, protection against challenge infection was partial in terms of reduction of virus shedding and clinical disease early after infection (12, 47). As the ability to reduce virus shedding early after infection has been linked to the presence of cell-mediated immunity (3, 25, 47), we have attempted to enhance antiviral T-cell responses by the inclusion of different adjuvants.

In this study, we tested plasmids encoding gamma interferon (IFN-γ) and interleukin-12 (IL-12), as these cytokines may act as adjuvants through stimulation of T-helper-1 or antigen-specific $CD8^+$ CTL responses (4, 6, 22, 35, 46). Furthermore, we tested the immunostimulatory properties of unmethylated CpG motifs, as present in the ampicillin resistance gene (37, 38, 40) of plasmid pUC18 (New England Biolabs). In addition, we tested lipophilic amine dimethyldioctadecylammonium bromide in water (DDA) and sulfolipo-cyclodextrin in squalene in water (SL-CD), two conventional adjuvants already shown to enhance the efficacy of conventional vaccines (5, 17, 19, 20, 21, 31, 32, 39).

To produce vectors encoding biologically active porcine IL-12 (pIL12), cDNA encoding the pIL12 p40 chain was excised by *Apa*I/*Sph*I digestion of pGEM3Z-IL12p40 (26) and ligated into *Eco*RV-digested VR1012 (VR-p40). Plasmid VR1012 contains the human cytomegalovirus immediate-early promoter, intron A, the processing signal for bovine growth hormone polyadenylation, and the gene encoding kanamycin resistance (15). The entire expression cassette from VR-p40 was isolated by *Apa*LI digestion and ligated into *Dra*I-digested VR1012, yielding VR-p40*. To obtain a vector that encodes both the p35 and the p40 chains, the cDNA encoding the p35 chain of pIL12 was excised by *Xho*I/*Sma*I digestion of pBluescriptIISK(-)IL12p35 and cloned into *Eco*RV-digested VR-p40* (VR-pIL12).

cDNA encoding porcine IFN-γ (pIFN-γ) was isolated by *Bam*HI/*Eco*RI digestion of pBS+Po IFN-γ 6-A1 and cloned into *Eco*RV-digested VR1012, yielding VR-pIFN-γ. Plasmids grown in the DH5α strain of *Escherichia coli*, purified on Qiagen columns (Qiagen), were transfected using Lipofectamine Plus (Gibco BRL) according the manufacturer's instructions. Culture supernatants of COS-7 cells collected 72 h posttransfection with VR-pIL12 were analyzed for pIL12 activity, using a previously described bioassay (7). Briefly, human peripheral blood mononuclear cells (PBMC) were incubated for 48 h in the presence of COS-7 supernatants, after which [^{3}H]thymidine incorporation was determined. As a positive control, recombinant human IL-12 (Roche) was used. As shown in Fig. 1, VR-pIL12 encoded biologically active pIL12, as culture supernatants from VR-pIL12-transfected COS-7 cells clearly stimulated PBMC proliferation, whereas supernatants from VR1012- or VR-p40*-transfected COS-7 cells did not.

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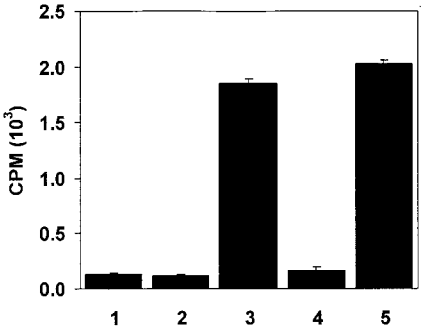


FIG. 1. Expression of biologically active pIL12 by transfected COS-7 cells. Culture medium samples of COS-7 cells transfected with the plasmid VR1012 (1), VR-p40 (2), or VR-pIL12 (3) were collected 72 h posttransfection, and the ability to stimulate the proliferation of human PBMCs was evaluated. Culture medium (4) and medium containing 12 ng of recombinant human IL-12/ml (5) were used as controls. Data are expressed as the geometric means + the standard errors of the means. Results shown are representative of two similar experiments.

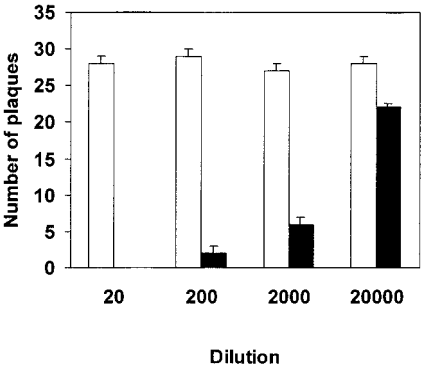


FIG. 2. Expression of biologically active pIFN-γ by transfected COS-7 cells. Culture medium samples of COS-7 cells transfected with VR1012 (white bars) or VR1012-p IFN-γ (black bars) were collected 72 h posttransfection, diluted 20, 200, 2,000, or 20,000 times, and assayed for antiviral activity, using an FMDV plaque reduction bioassay. Data are expressed as the geometric means + the standard errors of the means. Results shown are representative of two similar experiments.

Culture supernatants of COS-7 cells collected 72 h posttransfection with VR-pIFN-γ were analyzed for pIFN-γ activity using a pIFN-γ enzyme-linked immunosorbent assay (Biosource International) and in a foot-and-mouth disease virus (FMDV) plaque reduction bioassay. In the FMDV bioassay, secondary swine kidney (SK-2) cells, seeded in 6-well plates (Cellstar; Greiner, Frickenhausen, Germany), were incubated for 15 min with twofold dilutions of the samples, after which FMDV strain C_1 Detmold was added. After 1 h, medium containing 1% (vol/vol) methylcellulose was added, and after 48 h, wells were rinsed with 1% citric acid and stained with Amidoblack.

VR-pIFN-γ encoded pIFN-γ, as we could detect amounts of 1 to 2 μg of pIFN-γ/ml in supernatants of VR-pIFN-γ-transfected COS-7 cells in the enzyme-linked immunosorbent assay, whereas we could not detect any pIFN-γ in supernatants of VR1012-transfected COS-7 cells. In addition, the encoded pIFN-γ was biologically active, as it reduced FMDV replication (Fig. 2).

To evaluate the immunogenicity of different DNA vaccine-adjuvant combinations, 10- to 12-week-old Dutch landrace pigs from the specified-pathogen-free herd of ID-Lelystad were vaccinated. Pigs born from unvaccinated sows and free from antibodies against PRV prior the start of the experiment were vaccinated three times at intervals of 4 weeks. At each vaccination, 2 ml of the vaccine preparation was injected intradermally using a 22-gauge needle. Groups of four pigs received combinations of VR-gB and VR-gD (400 μg each) in the absence or presence of either VR-pIFN-γ, VR-pIL12, or pUC18 plasmid (400 μg each) or in the presence of SL-CD (16 mg of sulfolipo-cyclodextrin, 40 mg of Tween 80, and 160 mg of squalane per ml [17]) or DDA (16 mg per ml; Aldrich). Control pigs were vaccinated with 800 μg of plasmid VR1012. All experimental procedures and animal management procedures were undertaken in accordance with the requirements of the animal care and ethics committees of the institute. At weekly intervals, starting 1 week prior to the first vaccination, blood samples were collected to assess the presence of VN antibodies and CMI responses. VN antibodies were detected using a method previously described (2). Briefly, heat-inactivated serum was incubated with 100 50% tissue culture infective doses ($TCID_{50}$) of PRV strain NIA-3 for 24 h at 37°C, after which SK-6 cells were added. Titers are expressed as $\log_{10}$ values of the reciprocal of the highest serum dilution inhibiting cytopathogenic effect in 50% of the cell cultures. As shown in Fig. 3, all pigs developed VN antibodies after the second vaccination with VR-gB and VR-gD. Codelivery of pIFN-γ DNA, pIL12 DNA, SL-CD, or pUC18 did not enhance VN antibody responses. In contrast, codelivery of DDA significantly enhanced the induction of VN antibodies ($P \leq 0.05$) after the second and third vaccinations. Analysis of immunoglobulin 1 and immunoglobulin 2 isotype-specific antibody responses (24) confirmed the observation that only codelivery of DDA significantly enhanced the PRV specific antibody response (E. M. A. van Rooij, unpublished observations).

Lymphocyte proliferative (LPT) responses were determined as previously described (48) and expressed as stimulation index (SI) values (calculated as the number of counts [mean of quadruplicate wells] of PRV-stimulated PBMC divided by the number of counts [mean of quadruplicate wells] of mock-stimulated PBMC). As shown in Fig. 4, pigs vaccinated in the presence of DDA developed already clear LPT responses after the first vaccination, whereas the other DNA-vaccinated groups developed PRV-specific LPT responses only after the third vaccination. Furthermore, codelivery of DDA resulted in significantly ($P \leq 0.05$) higher LPT responses than those for pigs vaccinated with VR-gD and VR-gB alone, whereas codelivery of the other adjuvants did not significantly enhance PRV-specific LPT responses.

Six weeks after the third vaccination, pigs were challenged intranasally with 10^5 PFU of virulent wild-type strain NIA-3 per animal to assess the level of protection obtained by vaccination (34). To determine viral replication, swab specimens of oropharyngeal fluid were titrated on SK-6 monolayers as previously described (23). All groups of pigs vaccinated with VR-gB and VR-gD excreted PRV for a significantly ($P \leq 0.05$)

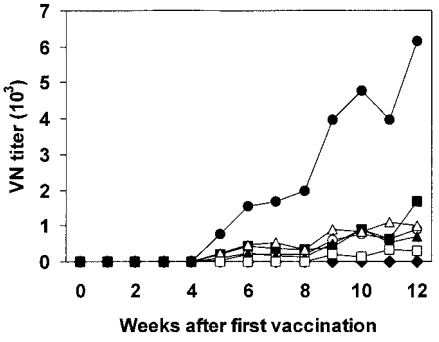


FIG. 3. Analysis of PRV-neutralizing antibodies in sera of immunized pigs. Pigs were vaccinated at weeks 0, 4, and 8 with VR1012 (♦), VR-gB + VR-gD (▲), VR-gB + VR-gD with pUC 18 (△), VR-IFN-γ (■), VR-pIL12 (○), SL-CD (□), or DDA (●). Samples from the individual pigs were tested. Data are expressed as geometric mean titers of the different groups. Differences in group averages were tested for statistical significance by a parametric one-way analysis of variance (ANOVA) (95% significance level) for the entire observation period after each vaccination and not for the individual time points. For reasons of clarity, error bars are not shown.

shorter period than sham-vaccinated control pigs (Fig. 5A). Compared to vaccination with VR-gB and VR-gD alone, codelivery of DDA significantly ($P \leq 0.05$) reduced levels of virus excretion during the period of peak excretion (days 2 to 5) after challenge (Fig. 5A). In addition to the effect seen with codelivery of DDA, codelivery of SL-CD also significantly reduced peak levels of virus excretion, although significantly less than DDA did.

The challenge infection with virulent PRV resulted in severe clinical signs in sham-vaccinated control pigs (nasal discharge,

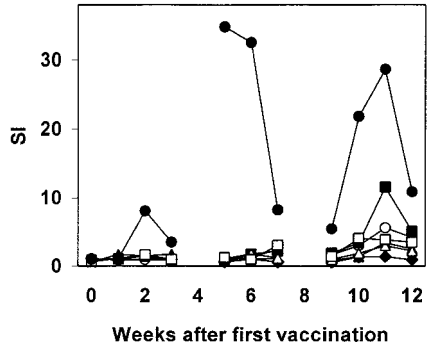
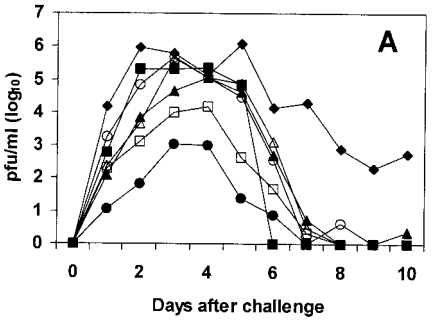


FIG. 4. Induction of PRV-specific T-cell responses in vaccinated pigs. Pigs were vaccinated at weeks 0, 4, and 8 with VR1012 (♦), VR-gB + VR-gD (▲), VR-gB + VR-gD with pUC 18 (△), VR-p IFN-γ (■), VR-pIL12 (○), SL-CD (□), or DDA (●). PBMCs were stimulated for 4 days with medium or live PRV, after which [^{3}H]thymidine incorporation levels were determined. Data are expressed as SI values. Based on the SI values of the control group (mean + 3× standard deviation), an SI of ≥2.5 was considered positive. Throughout the experiments, counts of mock-stimulated PBMCs ranged from 300 to 1,500 and standard errors of the means of quadruplicates were less than 20%. Samples from the individual pigs were tested. Data are expressed as geometric mean SI values of the different groups. Differences in group averages were tested for statistical significance by a parametric one-way ANOVA (95% significance level) for the entire observation period after each vaccination and not for the individual time points. For reasons of clarity, error bars are not shown.



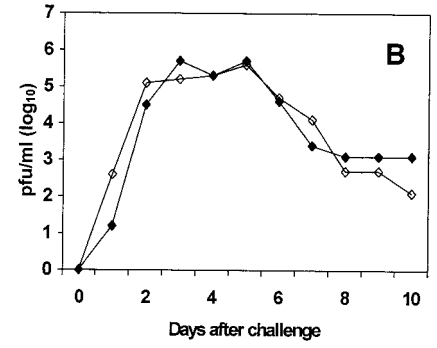


FIG. 5. Virus excretion after challenge infection with PRV strain NIA-3. (A) Pigs vaccinated with VR1012 (♦), VR-gB + VR-gD (▲), VR-gB + VR-gD with pUC 18 (△), VR-p IFN-γ (■), VR-pIL12 (○), SL-CD (□), or DDA (●) were challenge infected 6 weeks after the third vaccination. Samples from the individual pigs were tested. Data are expressed as geometric mean virus titers ($\log_{10}$) per gram of oropharyngeal fluid for the different groups. Differences in group averages were tested for statistical significance by a parametric one-way ANOVA (95% significance level). For reasons of clarity, error bars are not shown. (B) Pigs vaccinated with VR1012 (♦) or VR-IE with DDA (◇) were challenge infected 6 weeks after the third vaccination. Samples from the individual pigs were tested. Data are expressed as geometric mean virus titers ($\log_{10}$) per gram of oropharyngeal fluid for the different groups. There were no significant differences in results between VR-IE- and sham (VR1012)-vaccinated pigs.

coughing, ataxia, and convulsions; one pig died). In all groups, vaccination with VR-gB and VR-gD significantly ($P \leq 0.05$) shortened the duration of these clinical signs (Fig. 6A). According to the clinical signs, pigs vaccinated in the presence of DDA, pIFN-γ DNA, or pIL-12 DNA suffered for a significantly shorter period ($P \leq 0.05$) than pigs vaccinated with VR-gB and VR-gD alone. Only pigs vaccinated in the presence of DDA remained free of clinical signs (Fig. 6A), and theirs was the only group that exhibited significantly ($P \leq 0.05$) better growth performance compared to those of the others, as assessed by calculating the mean relative daily gain (MRDG) in body weight during the first week after challenge (45) compared to that of the sham-vaccinated control pigs (Fig. 6B).

This study demonstrates that DDA greatly improved PRV-specific humoral immune responses and CMI responses after DNA vaccination. The adjuvanticity of DDA may be the result of the induction of an influx of antigen-presenting cells (11, 18), the production of cytokines such as interferons and interleukin-1 (28), or the enhancement of plasmid DNA transfec-

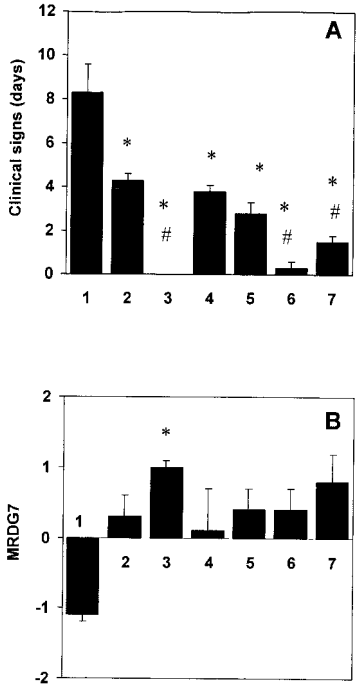


FIG. 6. Number of days of clinical signs (A) and growth performance (B) after challenge infection with PRV strain NIA-3. Pigs vaccinated with VR1012 (lane 1), VR-gB + VR-gD (lane 2), VR-gB + VR-gD with DDA (lane 3), SL-CD (lane 4), pUC18 (lane 5), VR-pIL12 (lane 6), or VR-p IFN-γ (lane 7) were challenge infected 6 weeks after the third vaccination. Clinical signs were recorded blindly for 14 days after infection. Growth performance during the first week after challenge was assessed by calculating the MRDG in body weight. For the duration of clinical signs, differences in group averages were tested by the nonparametric Kruskal-Wallis test. For the MRDG, differences in group averages were tested for statistical significance by a parametric one-way ANOVA. Significance levels were set at 95%. #, results significantly different from those for pigs vaccinated with VR-gB plus VR-gD alone (lane 2); *, results significantly different from those for the sham-vaccinated control pigs (lane 1).

tion efficacy (14). Codelivery of DDA also clearly improved protection against PRV challenge, but although clinical signs were absent, protection was not complete, as pigs still excreted virus and suffered some growth retardation (MRDG ± 1; this value was significantly different from that for sham-vaccinated pigs but not from those for other groups of DNA-vaccinated pigs [compare the minimal requirement for PRV vaccines, as specified by the European Pharmacopoeia, of >1.5). It has been suggested that adjuvants like DDA or CpG motifs themselves provide protection against infections (e.g., those caused by *Listeria monocytogenes*) through activation of macrophages (11, 36). However, taking into consideration our experimental design (intradermal vaccination and intranasal challenge infection, with a time period of 6 weeks between vaccination and challenge infection) and the observed enhanced PRV-specific immune responses, we speculate that DDA exerts its protective effect mainly through enhancement of the PRV-specific immune responses. This speculation is supported by the fact that in a similar experiment, we demonstrated that a vaccine consisting of DNA encoding the PRV immediate-early gene (VR-IE) in combination with DDA did not provide protection against challenge infection. Pigs vaccinated with VR-IE plus DDA did not develop PRV-specific VN antibodies nor PRV-specific LPT responses and were not protected against challenge infection, as exemplified by the lack of significant reduction in virus-shedding levels compared to those for sham-vaccinated control pigs (Fig. 5B), indicating that DDA (in combination with a nonprotective DNA vaccine) does not provide protection. Furthermore, adjuvants like DDA or CpG motifs themselves have also been shown not to protect against other viral infections, like Semliki forest virus and lymphocytic choriomeningitis virus infections (27, 36).

Remarkably, the other agents tested in this study did not exert significant adjuvanticity. Plasmid pUC18 may have lacked adjuvanticity because of species-specific recognition of CpG motifs (1, 16, 33). Although codelivery of plasmids encoding IL-12 has been shown to augment antigen-specific $CD4^+$ T-helper-1 (4, 42, 43, 46, 50) and $CD8^+$ CTL (4, 13, 22, 35, 46) responses, other studies did not reveal immune stimulating effects or even observed adverse effects (9, 10, 29). Similarly, notwithstanding the fact that codelivery of plasmids encoding IFN-γ has shown to enhance immunity (4, 6, 41), others (44) were unable to demonstrate significant effects of codelivery of IFN-γ DNA. Although we could not demonstrate a significant effect of the codelivery of pIFN-γ DNA or pIL12 DNA on antigen-specific immune responses, we observed that codelivery of pIFN-γ DNA or pIL12 DNA reduced the occurrence of clinical signs (without having any detectable effect on levels of virus shedding). Despite the fact that the underlying mechanism is not clear, similar observations have been made for IFN-γ. Codelivery of recombinant pIFN-γ during vaccination of pigs with inactivated PRV did not affect nasal virus excretion but diminished disease parameters such as fever and loss of body weight (49), and IFN-γ protected mice against fatal herpes simplex virus-induced encephalitis without reducing virus replication (8, 30).

In summary, codelivery of DDA during DNA vaccination against PRV enhanced the induction of antigen-specific humoral and cell-mediated immunity and improved protection against the challenge infection in terms of virus shedding and clinical signs. These results indicate that the efficacy of DNA vaccines can be improved by the conventional adjuvant DDA.

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